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13. ABSTRACT (Maximum 200 Words)

Blood cholinesterase (ChE) biomarkers are an important way to monitor exposure to anticholinergic pesticides and chemical warfare (CW) agents and to establish whether some are at greater risk tan others form exposure to them. Many clinical and research laboratories use the colorimetric Ellam assay based on the hydrolysis of acetylthiocholine. CHPPM (US Army Center For Health Promotion and Preventive Medicine) uses the Michel delta pH method to monitor more than 25,000 DoD personnel each year. Paired samples of RBCs assayed by CHPPM were sent to UC Davis to be assayed with acetylthiocholine by the Ellman method. Slopes of pH vs Ellman results for two sets of samples had poor correlations of r^2 of 0.64 and 0.14. Sample sets were assayed on day of receipt and the following to check on the timing of assay after shipping as a possible cause of the poor correlation. No effects were observed. Parallel work established conversion factors for clinical laboratories, assisting them in meeting California regulations and being approved for occupational ChE monitoring. Future work will involve Ellman and delta pH assays carried at the UC Davis laboratory to reduce variability as a next step in establishing a conversion between assays.

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INTRODUCTION

There is a need for rapid, high throughput, reliable and transferable determinations of blood cholinesterase (ChE) levels to provide early warning of exposures due to the intensive use of pesticides such as organophosphate esters (OPs) and threats of chemical warfare agents. The colorimetric Ellman assay based on the hydrolysis of acetylthiocholine (Ellman, *et al.*, 1961) is used by many clinical and research laboratories. A slower delta pH method based on that of Michel (1949) is used to monitor more than 25,000 DOD personnel each year by the US Army Center For Health Promotion and Preventive Medicine (CHPPM). Although pH assays are reliable and have low variability, they are not readily adaptable for automation or field use.

One goal of this project is to establish a conversion factor between the pH and colorimetric assays applicable to monitoring studies and field tests. Another goal was to provide conversion factors for the portable Test-Mate kit manufactured by EQM, Inc. since studies have shown that the current model does not adequately adjust for temperature to be useful for field use. Plans agreed to by the manufacturer are to use a new model from the manufacturer with improved assay parameters. A bovine RBC AChE standard preparation we developed is used as a reference standard in assay comparisons.

Another issue is that of genetically sensitive individuals exposed to anticholinergic chemicals. Lowered butyrylcholinesterase (BChE), a scavenger of antiChE agents, may put individuals at increased risk from OP and CB agents (reviewed by Wilson, 1999) and a polymorphic form of paraoxonase (PON1), which destroys selected OPs, has been reported to be reduced in a cohort of veterans suffering from "Gulf War Syndrome" (Haley *et al.*, 1999). There is evidence that low levels of BChE and PON1 affect sensitivity to OP exposures of experimental animals (Shih *et al.*, 1998, Broomfield *et al.*, 1991). Following completion of the cholinesterase tasks, plans are to investigate the incidence of lowered BChE and PON1 in the blood samples.

BODY

Materials

All chemicals were purchased from Sigma Chemical Co.

Methods

Sample Handling

RBC samples are shipped overnight on cold packs by CHPPM personnel. Upon receipt at UC Davis, the temperature of the samples is checked (CHPPM protocol states samples will be <10°C during shipping). The samples are stored at 4°C and kept on ice during use. Ghost RBC samples are stored at -70°C, and shipped overnight on dry ice to CHPPM from UC Davis.

ChE Determinations

RBC samples received from CHPPM are diluted 1/50 in a lysis buffer (0.5% Triton X-100, 0.1 M sodium phosphate, pH 8) and their ChE activities measured using the colorimetric method of Ellman, *et al.* (1961), modified for use with an automatic microplate reader. Activity is determined in the presence and absence of 0.02 mM quinidine sulfate, a selective BChE inhibitor.

Ghost RBC Preparation
Bovine blood is centrifuged at 1000 x g and the plasma discarded. The RBCs are resuspended in isotonic buffer and washed twice. RBC ghosts are prepared by lysing the cells with hypertonic buffer. The membrane bound AChE is centrifuged at 100,000 x g and the pellet solubilized in buffer with Triton X-100 detergent. The solution is diluted and stored at -70°C (Arrieta *et al.*, 2003).

Task One.
Comparisons: The first task is to conduct a careful comparison of the Ellman assay performed under optimum conditions and the DOD pH assay to examine the variability and reliability of both assays, to establish baseline values and to generate conversion factors to enable comparisons between them and with other proposed or commercial assays. Last year five sets of RBC samples were sent from CHPPM to UC Davis. This year several more comparisons were undertaken (Figures 1-4). Although several sets showed good correlations and similar slopes between the laboratories, some did not (Tables 1 and 2). There was a concern about when the samples were assayed at the UC Davis laboratory and if a delay of one day affected the comparison. The latest sample sets were assayed on the day of receipt and the following day. There does not appear to be any effect on the correlation due to the timing of the assay (Table 2). Our next step is to do direct comparisons of the Ellman and pH assays in our own laboratory, eliminating possible variability introduced by shipping samples.

Table 1. Comparison of AChE Measurements at CHPPM and UC Davis

Sample Set	Slope	Correlation, r^2
1	0.047	0.74
2	0.045	0.79
3	0.028	0.25
4	0.048	0.80
5	0.025	0.34

Slopes are ratio of delta pH/Ellman AChE activities. Previously presented.

Table 2. Further Comparison of AChE Measurements at CHPPM and UC Davis

Sample Set	Slope	Correlation, r^2
6	0.039	0.64
6 Next Day	0.046	0.67
7	(-) 0.016	0.14
7 Next Day	(-) 0.027	0.24

Slopes are ratio of delta pH/Ellman AChE activities.

During this past year, in parallel with the work with CHPPM, we have been working with clinical laboratories in California to assist them in developing a conversion factor between their mandated ChE assays and our reference assay, with the collaboration of the California (CA) Department of Pesticide Regulation (DPR) and the NIOSH Western Center for Agricultural Health & Safety at UC Davis. The research, recently submitted for publication (Wilson *et al.*, 2004) serves as an example of the studies we are doing with CHPPM. CA has a long-standing formal blood ChE monitoring program for mixers, loaders and applicators of pesticides.

When we found commercial clinical kits were not optimal for assaying blood ChEs, CA regulations were revised to specify use of the Ellman ChE assay or to demonstrate a conversion factor with a correlation (r^2) of 0.9 or better. When we were first enlisted by DPR to work with the clinical laboratories in 2000, only 2 of 7 participating laboratories generated an acceptable correlation for red blood cells; only 4 of 5 laboratories had an acceptable correlation for plasma ChE. Last year, when DPR restated the need to meet this requirement, we worked with several of the clinical laboratories using our bovine ghost RBC ChE as a reference. But, only 3 of 10 laboratories had acceptable correlations. Next, we provided all interested laboratories with human blood and plasma samples to perform a comparison study. Fourteen laboratories participated; 9 met the ChE criteria for whole blood, 14 for plasma and 6 for RBC. Based on such data, on July 8, 2003, DPR notified the CA Agricultural Commissioners that only 9 of the participating laboratories were approved for ChE testing. Later work resulted in acceptable RBC values for 2 of the laboratories and their approval. We continue to work with laboratories interested in being on the approved list.

Normal Range of AChE Levels: The availability of such a large number of RBC AChE samples from CHPPM provides an important opportunity to establish a normal range of human cholinesterase values better than has been done before and to convert the delta pH values to Ellman colorimetric ranges once the conversion part of Task One has been accomplished.

With the collaboration of epidemiologist-physician Dr. Stephen McCurdy, (UCD) Blood specimens from 991 Department of Defense personnel without a potential exposure to anti-cholinesterase agents were statistically analyzed. The median age was 43 years (range 18-76). 823 specimens (82.1 %) were from men. Men were on average older than women (median age 44 vs. 37 years, $p<0.001$, Wilcoxon test). The mean $\pm$ SD for delta pH was 0.75 ± 0.06 units. Delta-pH values were greater for men than for women (0.74 vs. 0.73, $p<0.001$, Wilcoxon test). Multivariate linear regression analysis showed an association for delta pH with age (slope +0.0008 delta-pH units for each year of age, $p<0.001$). There was a small, but statistically significant, reduction in delta pH associated with time (-0.006 delta pH units per 100 days, $p<0.001$). A multiple regression model incorporating age, gender, and test date explained only 3.4% of the observed variance. The small magnitude of these effects and their minimal role in accounting for the observed variability suggest it appropriate to ignore such factors when evaluating delta-pH data. The next step in our project is to correlate the delta pH values with those from

the more commonly used colorimetric Ellman test. The results were reported at two meetings and are being prepared for publication (McCurdy *et al.*, 2003, 2004).

Task Two. Testing the stability and usability of a red blood cell ghost standard suitable for clinical standardizations has been carried out and the results published (Arrieta *et al.*, 2003). We have found that the activity of the preparation is too low for good detectability by the delta pH method. Plans are to prepare a standard with higher activity and retest again.

The ghost RBC standard has been useful in previous work on ChE method comparisons, such as with the Test-mate Kit (Oliveira, *et al.*, 2002), and a sample is included with each run conducted at UC Davis.

Task Three. The third goal is to conduct experiments with a specially designed Test-mate Kit with an uncorrected read out to establish the conditions for an optimum assay and construct conversion factors to harmonize its results with clinical laboratory assays. The new instrument did not become available this year. A letter from Patrick Eberly, CEO of EQM Inc. is attached as an appendix to this report. He informs us that a new, temperature regulated model is nearing the end of development but that there have been delays readying it for testing.

Task Four. The feasibility of incorporating BChE variant and PON1 polymorphisms into a screen of workers, for whom blood ChE baselines are required, using a selected set of DOD personnel will be addressed later in the project.

KEY RESEARCH ACCOMPLISHMENTS

- Correlations of RBC activity measured by the Ellman method and by the delta pH method continue and sources of variability are being investigated.
- AChE activity levels in the current samples are in agreement with the existing CHPPM data set.
- Older delta pH data are proving useful in establishing normal ranges of human RBC AChE activity.
- A bovine ghost RBC AChE standard is in use for Ellman assays and a higher activity standard will be prepared for the delta pH assays.

REPORTABLE OUTCOMES

S.A. McCurdy, J.D. Henderson, D.E. Arrieta, L.J. Lefkowitz, R.E. Reitstetter, and B.W. Wilson. 2004. Determining a reference value for blood cholinesterase using Defense Department personnel. Abstract To Be Presented at SOT Meeting, March 2004

D. Arrieta, A. Ramirez, E. DePeters, D. Bosworth, and B.W. Wilson. 2003. Bovine red blood cell ghost cholinesterase as a monitoring standard. Bulletin of Environmental Contamination and Toxicology 71(3), 447-452.

B. W. Wilson, J. D. Henderson, D.E. Arrieta, and M.A. O'Malley. 2003. Assisting clinical laboratories meet requirements of cholinesterase monitoring in California. Submitted to Int. J. of Toxicology

S.A. McCurdy, J.D. Henderson, D.E. Arrieta, L.J. Lefkowitz, R.E. Reitstetter, and B.W. Wilson. 2003. Normal range of cholinesterase levels among US Defense Department personnel. Poster presented at NIOSH Conference, November, 2003 San Francisco, CA.

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CONCLUSIONS

The first stage of establishing a conversion factor between the delta pH and Ellman assays has been completed and we are seeking to find and correct the sources of variability noted.

A statistical analysis of a set of CHPPM data has been completed, generating a distribution of AChE levels that provide the first step towards a reliable range of unexposed human RBC AChE levels useful for comparing specimens from suspected exposure episodes.

Work is proceeding on the major tasks. Unanticipated delays due to turnover of leadership at CHPPM (replacement of Capt. Reitstetter by Capt. Lefkowitz) are behind us and both scientists will continue to collaborate on the project. Capt. Lefkowitz has sent us equipment used at CHPPM. Conducting both pH and Ellman assays at UC Davis should speed up the comparisons. We await completion of the new model of the Test-Mate kit by EQM Inc.

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APPENDICES

Figure 1. RBC Cholinesterase Assay Comparison: Sample Set 6

Figure 2. RBC Cholinesterase Assay Comparison: Sample Set 6, Next Day

Figure 3. RBC Cholinesterase Assay Comparison: Sample Set 7

Figure 4. RBC Cholinesterase Assay Comparison: Sample Set 7, Next Day

Letter from EQM, Inc.

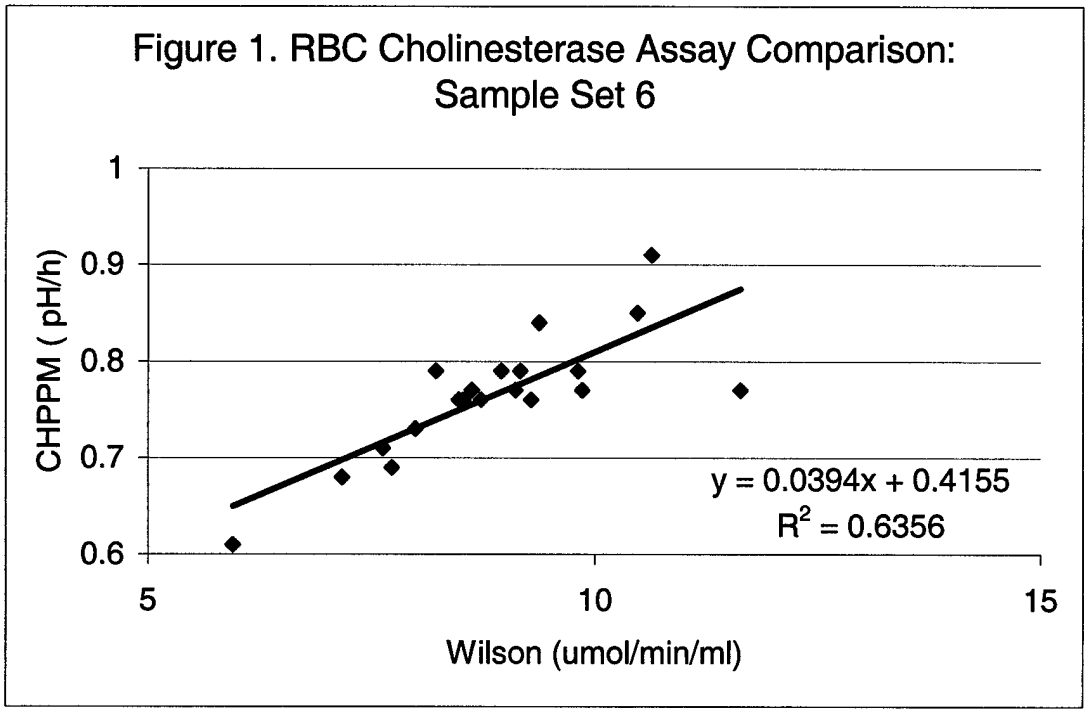
Figure 1. RBC Cholinesterase Assay Comparison:
Sample Set 6
CHPPM (pH/h)
1
0.9
0.8
0.7
0.6
y = 0.0394x + 0.4155
R2 = 0.6356
5
10
15
Wilson (umol/min/ml)

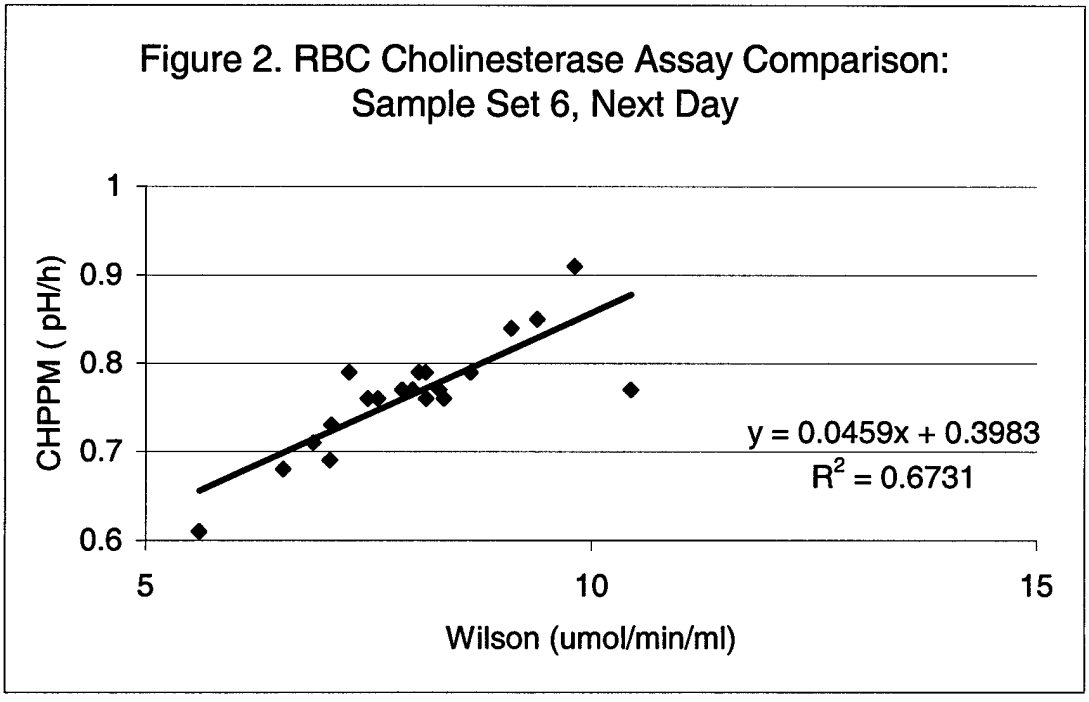
Figure 2. RBC Cholinesterase Assay Comparison:
Sample Set 6, Next Day
CHPPM (pH/h)
1
0.9
0.8
0.7
0.6
y = 0.0459x + 0.3983
R2 = 0.6731
5
10
15
Wilson (umol/min/ml)

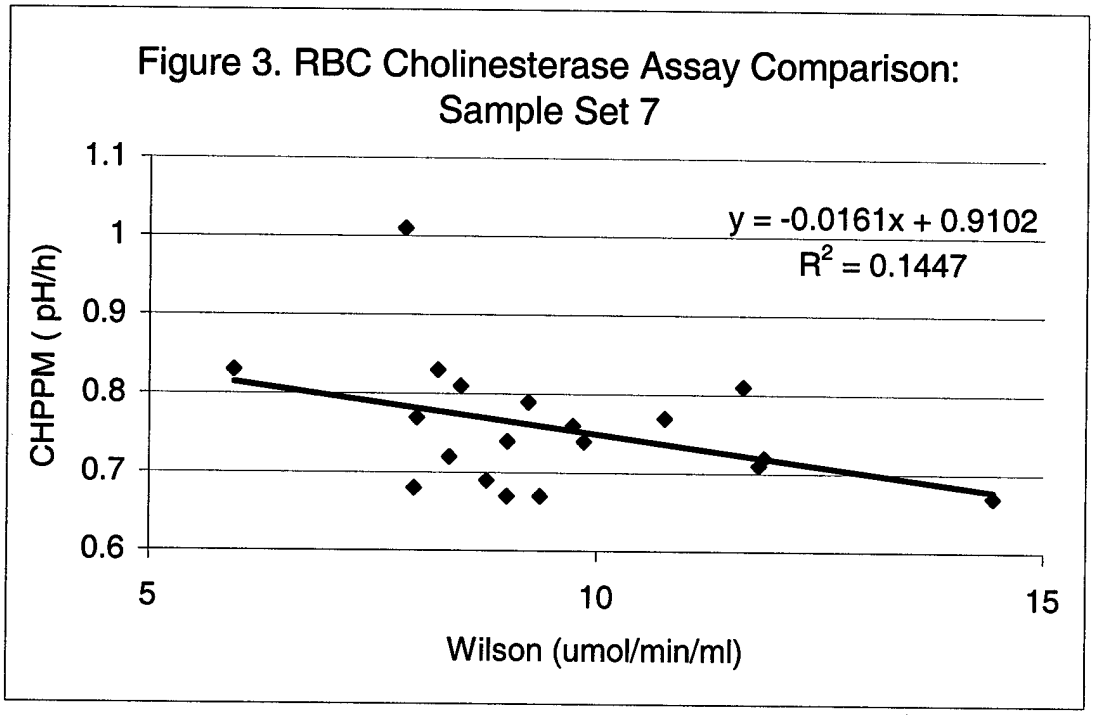
Figure 3. RBC Cholinesterase Assay Comparison:
Sample Set 7
y = -0.0161x + 0.9102
R2 = 0.1447
CHPPM (pH/h)
1.1
1
0.9
0.8
0.7
0.6
5
10
15
Wilson (umol/min/ml)

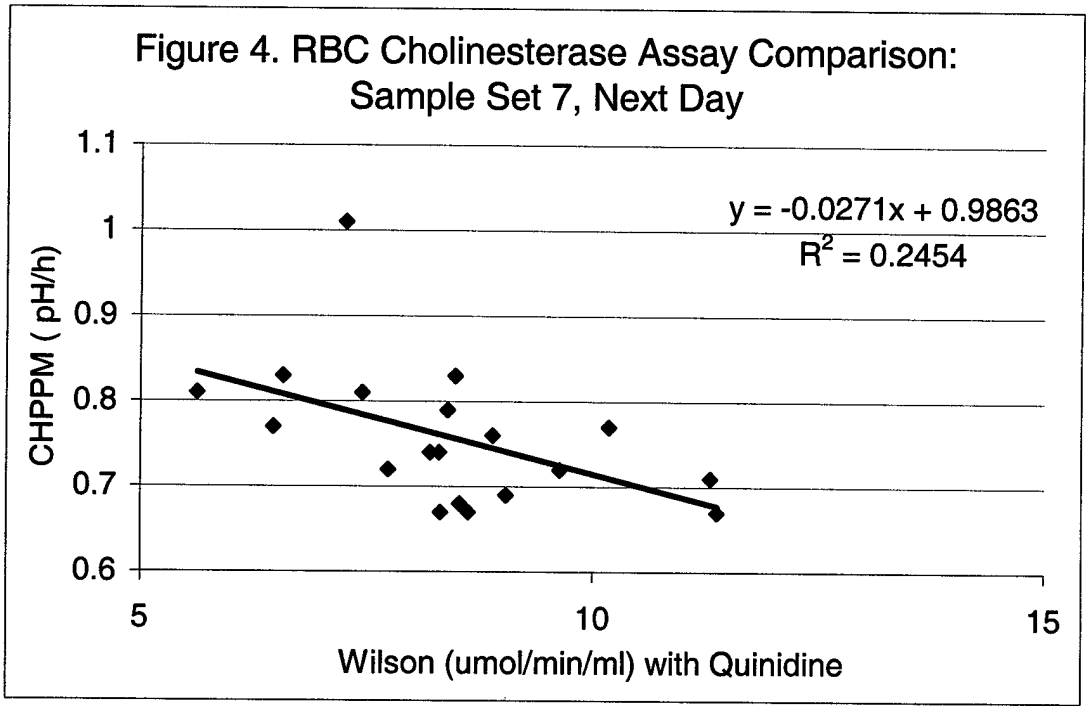
Figure 4. RBC Cholinesterase Assay Comparison:
Sample Set 7, Next Day
y = -0.0271x + 0.9863
R2 = 0.2454
CHPPM (pH/h)
1.1
1
0.9
0.8
0.7
0.6
5
10
15
Wilson (umol/min/ml) with Quinidine

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13 October, 2003

Dear Professor Wilson:

Thank you for your continued interest in the Test-mate ChE Cholinesterase Test System. As you requested, I am writing this letter to be included with your annual report that will be submitted to USACHPPM this week. The following is a brief summary of recent research, development, and engineering accomplishments.

The new Test-mate ChE (Version D) will be offered for commercial sale in the near future. The Version D instruments are far superior to the Version C instruments that are now being sold. The new instruments will contain several design improvements that include the extension of the operational temperature range for accurate measurements of AChE, Hgb, and Q (hemoglobin corrected erythrocyte cholinesterase) to allow operation between 10ºC and 50ºC. As in the older Version C instruments, the Version D instruments are currently programmed to display testing results automatically adjusted to 25ºC. Like the previous versions of the Test-mate ChE, the new Test-mate ChE (Version D) instruments are intended for use only with human blood. Four such new prototype instruments have been tested - two by the German Armed Forces and two by USAMRICD.

The Cholinesterase Chemistry Set (manufactured by EQM Research, Inc.), is a moderately priced collection of reagents for use in the accurate determination of AChE, BChE, and Hgb using a Molecular Devices SpectraMax microplate reader, will soon be offered for sale. This system has been extensively tested and additional testing is being scheduled. The Cholinesterase Chemistry Set presents the final results as read at the assay reaction temperature of 37ºC. A detailed manuscript will be submitted to the Bioscience 2004 Review Committee.

Prior to its formal release, I am currently engaged in changing the Test-mate ChE (Version D) to display results directly comparable to The Cholinesterase Chemistry Set microplate method. This will provide consistent laboratory cholinesterase measurements and field cholinesterase measurements. Once this composite package is fully completed and more thoroughly tested, I will be glad to arrange for both a Test-mate ChE (Version D) and The Cholinesterase Chemistry Set reagents to be available to you and your laboratory personnel for evaluation.

Best Regards,

J. Patrick Eberly

J. Patrick Eberly, Ph.D.
President - EQM Research, Inc